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THE EFFECT OF TEMPERATURE ON THE SORPTION OF ORANGE II BY WOOL[1]

BY BARBARA BENSON, JOAN KRUGER, RITA DONOVAN, AND P. LAROSE

ABSTRACT

The sorption of Orange II dye by wool from acid solutions was measured at various temperatures between room temperature and the boiling point. Most of the measurements were carried out in 0.0078 *M* sulphuric acid solutions, made 0.0062*M* with respect to sodium sulphate but an isotherm at 60°C. was determined without the use of salt in the solution. The change in equilibrium with temperature is so rapid that appreciable changes can be observed within a few minutes. The equation representing the relationship between the amount sorbed at equilibrium was found to be of the same form at 60°C. as that previously reported for 100°C. The energy changes accompanying the sorption of Orange II were calculated by assuming that the Gilbert–Rideal equations hold.

INTRODUCTION

The published data concerning the effect of temperature on the sorption of acids and acid dyes by wool are rather meager and not very convincing. Few investigators appear to have tackled this problem. Elöd and Silva (3) determined the amount of hydrochloric acid sorbed by wool at 20°C. and at 90°C. The results did not allow an accurate estimation of the difference in amount taken up by the wool at those two temperatures but, with an acid solution of initial pH = 2.6, the amount of acid sorbed was apparently 0.047 milliequivalent per gram greater at 20°C. than at 90°C. It may be noted, however, that these investigators did not obtain the same equilibrium points on absorption as on desorption.

Speakman and Smith (11) investigated the effect of temperature on the rate of diffusion of dyes into the wool fiber and in this connection they also carried out a few experiments with Acid Orange 2G to determine the influence of temperature on the amount sorbed by the wool. They noted a maximum sorption at 35°–40°C. They attributed this to the fact that the swelling of wool is a minimum at that temperature.

Smith and Harris (10) in studying the sorption of Orange II found a value of 0.85 milliequivalent per gram for the amount sorbed from the dye solution at the boiling point and practically the same value at 50°C. Ender and Müller (4)

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also obtained the same sorption of Orange II acid and other dye acids at 90°C. as at 50°C. Elöd and Reutter (2) measured the amount of Crystal Ponceau 6R sorbed at 40°, 50°, 60°, 70°, and 80°C. and found the same value for these various temperatures, but it is questionable whether equilibrium had been reached at the lower temperatures.

Goodall (6) has reported results of sorption experiments with Orange GG which indicate a somewhat smaller sorption at 100°C. than at 20° or 40°C. However, in a later paper with Hobday (7) a small increase in sorption was claimed for the same dye as the temperature was increased from 20°C. to 100°C. Experiments with Polar Yellow R by the same investigators gave results which showed a large temperature effect, but since the degree of dispersion of this dye is affected greatly by temperature, the results are not comparable with those obtained with a dye like Orange II which is regarded to be molecularly dispersed under ordinary dyeing conditions.

Steinhardt, Fugitt, and Harris (13) determined the effect of temperature on the titration curve of wool using hydrochloric acid at 0°, 25°, 40°, and 50°C. They found very little difference between the results obtained at 25°C. and those obtained at 40° and 50°C. but the sorption at 0°C. was a little higher. They also carried out measurements with Orange II at 0°, 25°, and 50°C. The results were practically the same at 0° and at 25° but at 50°C. the amount of dye acid sorbed was found to be lower. However, the measurements at 0° and 25° were not extended to solutions with a pH lower than 4.2 and these investigators assumed the same maximum combining capacity of 0.82 milliequivalent per gram at all temperatures although they obtained much higher values for the sorption at 50°C. in solutions with low pH.

It is evident from these results that further work on the sorption of acid dyes by wool at various temperatures is highly desirable. Since we had already begun a study of the acid dyeing mechanism with Orange II, experiments were carried out with this dye at various temperatures between room temperature and 100°C. to determine the change in the equilibrium between wool and solution as the temperature was varied.

METHODS

The procedure followed for measuring the amount of Orange II sorbed by wool from aqueous acid solutions at various temperatures was practically that followed in our earlier experiments dealing with the effect of acid strength and of salt on the sorption of the dye (1). The flask containing the dye solution and the wool was kept at the temperature desired by immersion in a constant temperature bath. The flasks were closed by means of rubber stoppers covered with tin foil after they had reached the temperature of the bath and they were stirred occasionally during the immersion period. In order to follow the course of the sorption with time, samples of the dye solution were removed from time to time for a colorimetric determination of the dye concentration, but these were replaced after examination in order to eliminate a correction for the dye removed with the sample. The amount of dye sorbed by the wool was calculated from the

change in the dye concentration of the dye bath as before. A determination of pH was also carried out on the dye solutions before and after the tests as in previous experiments.

All wool samples were equilibrated to a pH 5.0–5.5 before the tests. Most of the solutions were prepared with the purified dye but some of the experiments have been made with dye of 93% purity (determined by titanous chloride titration). However, as some of the earlier tests had shown, the small amount of impurities did not appear to affect the results, provided proper allowance was made for the dye content in calculating the dye concentration of the solutions.

The determination of the sorptive capacity of the wool was carried out after various times of immersion in order to judge when equilibrium had been reached. The equilibrium in many cases was attained in two different ways: first with undyed wool sorbing the dye to a maximum value which was taken as the equilibrium value, and second with wool which was dyed at the boiling point for one and one-half hours and which was then allowed to cool and stand in the bath at the desired temperature until a new equilibrium had been reached. A few tests were carried out in which the wool was dyed at 100°C. and then cooled rapidly or slowly in the dye bath before it was removed. All results represent the means of two, or in most cases three, replicate determinations.

RESULTS

To illustrate the order of magnitude of the changes noted with time, the two examples in Table I will suffice.

TABLE I

CHANGE IN DYE CONCENTRATION WITH TIME

Initial acid strength of bath, 0.0077*M* sulphuric acid.
Sodium sulphate concentration, 0.0062*M*.
Initial pH = 1.99.
C_B = concentration of dye in bath in milliequivalents per liter.
A = concentration of bath with originally undyed wool.
B = concentration of bath with wool initially dyed at 100°C.
C_F = dye sorbed in milliequivalents per gram of wool.

Temperature, °C.	30°		40°	
Time of immersion, days	C_B		C_B	
	A	*B*	*A*	*B*
1	0.035	0.0137	0.0624	0.0407
2	—		0.0445	0.0393
3	0.0176	0.0140	0.0416	0.0396
4	0.0168	0.0137	0.0405	0.0391
5	0.0162	0.0134	0.0394	0.0388
6	0.0168	0.0134	—	—
7	0.0160	0.0134	0.0431	0.0385
8	0.0157	—	0.0405	0.0376
9	—		0.0405	0.0376
10	0.0157			
11	0.0154			
12	0.0151			
Final pH	2.62	2.57	2.62	2.69
Final C_F	0.215	0.215	0.440	0.440

Table II summarizes the results obtained at various temperatures, but the effect of temperature on the equilibrium is best represented by Fig. 1 where the amount sorbed has been plotted against the dye concentration in the bath. For some of the temperatures, there is only one reference point for the corresponding curve and for that reason only a small section of the curve has been drawn but it will be clear from the course followed by the other curves where the curve for a particular temperature lies. Previous results obtained at 100°C. (1) have been included for comparison.

TABLE II

SORPTION EQUILIBRIUM VALUES AT DIFFERENT TEMPERATURES

Temp., ° C.	Acid concn.	Salt concn.	C_F	C_B		pH	
				A	*B*	*A*	*B*
30°	0.0078*M*	.0062*M*	0.178	0.0128	0.0120	—	2.72
"	0.0077	"	0.215	0.0151	0.0134	2.62	2.57
"	0.0077	"	0.431	0.0346	0.0271	2.62	2.75
40°	0.0076	"	0.178	0.0164	0.0159	2.64	2.67
"	0.0076	"	0.364	0.0385	0.0343	2.64	2.68
"	0.0078	"	0.440	0.0405	0.0376	2.62	2.69
"	0.0193	"	0.430	0.0360	—	1.86	—
"	0.0193	"	0.430	0.0357	—	1.89	—
50°	0.0076	"	0.416	0.0619	0.0542	2.63	2.70
"	0.0077	"	0.438	0.0588	0.0556	2.65	2.70
"	0.0077	"	0.438	0.0607	0.0567	2.67	2.70
"	0.0193	"	0.429	0.0506	—	1.89	—
60°	0.0078	"	0.310	0.0798	0.0754	—	—
"	0.0078	"	0.417	0.0963	0.0913	2.66	2.67
"	0.0078	"	0.418	0.0918	0.0900	2.67	2.69
"	0.0077	"	0.426	0.1045	—	2.70	—
"	0.0193	"	0.427	0.0814	—	1.91	—
"	0.0076	"	0.640	0.226	0.214	2.74	2.78
70°	0.0077	"	0.421	0.201	—	2.70	—
"	0.0183	"	0.425	0.142	—	1.92	—
100°	0.0077	"	0.400	0.569	—	2.58	—
60°	0.0076	No salt	0.218	0.0300	0.0288	2.53	2.60
"	"	"	0.436	0.0637	0.0617	2.60	2.60
"	"	"	0.559	0.107	0.109	2.99	2.77
"	"	"	0.642	0.172	0.166	2.83	2.85
"	"	"	0.780	0.386	0.393	3.22	3.27
70°	"	"	0.215	0.0468	0.0468	2.64	2.61

Table III shows the pH change accompanying the sorption of dye for relatively short periods of time at room temperature while Table IV gives the results of similar experiments carried out at 95° and 30°C. with a sulphuric acid solution without dye.

The effect of temperature on the sorption was also demonstrated by boiling the dye solution with the wool under reflux for one and one-half hours and then letting the solution cool under various conditions before removing the wool and measuring the dye sorbed by it. Table V gives the results of experiments in which the dye solution was allowed to cool slowly after boiling and the wool was removed when the solution had reached various temperatures. To obtain the

TABLE III

CHANGES OF pH ACCOMPANYING DYE SORPTION BY WOOL AT ROOM TEMPERATURE (23°-25°C.)

Initial acid strength	0.00382 *M*			0.01527 *M*		
Initial pH	1.93			1.72		
Time	C_F	C_B	pH	C_F	C_B	pH
5 min.	—	—	2.24			1.78
45 "	—	—	2.50			1.81
75 "	—	—	2.57			1.82
105 "	0.049	5.43	2.62	0.049	5.43	1.83
220 "	—	5.43	2.68	—	—	1.84
340 "	—	—	2.82	—	—	1.84
23 hr.	0.075	4.98	3.18	0.075	4.98	1.85
26 "	—	—	3.28	—	—	1.93
47 "	—	—	3.48	—	—	1.92
77 "	0.249	1.96	3.75	0.272	1.55	1.93

TABLE IV

SORPTION OF SULPHURIC ACID BY WOOL FOR VARIOUS PERIODS OF IMMERSION AT TWO DIFFERENT TEMPERATURES

Initial strength of acid 0.00773 *M*, pH 1.93

Temperature, °C.	Time	C_F	C_B	pH
95°	1 hr.	0.603	4.99	2.37
"	1½ "	0.610	4.88	2.38
"	2 "	0.612	4.84	2.39
"	5 "	0.612	4.84	2.48
"	22 "	0.617	4.75	2.60
"	29 "	—	—	2.66
30°	2 days	0.611	4.86	2.40
"	3 "	0.606	4.94	2.40
"	4 "	0.608	4.92	2.41

TABLE V

SORPTION OF DYE AT VARIOUS TEMPERATURES DURING COOLING OF DYE BATH AFTER A PRELIMINARY BOILING PERIOD

Initial strength of sulphuric acid 0.0084 *M*

Time, min.	Temperature, °C.	pH	C_B	C_F
0	100°	2.41	0.308	0.416
1	96°	—	0.283	0.418
2	92°	2.41	0.248	0.420
6	87°	—	0.208	0.422
8	82°	2.42	0.171	0.424
14	75°	2.44	0.133	0.426
19	70°	2.45	0.107	0.428
24	65°	2.46	0.081	0.429
32	60°	2.46	0.074	0.429
42	55°	2.46	0.063	0.430

results of Table VI, the solution was cooled rapidly under running tap water before the wool was removed at the temperature indicated. Other tests were carried out in which the wool was removed from the solution at the boiling point and the solution then allowed to cool to room temperature after which the wool was put back into the dye solution for different times. The results of these tests are given in Table VII.

It must be noted that the results of Tables V, VI, and VII, except those corresponding to 100°C., do not represent equilibrium conditions and that the dye bath concentrations are higher, and the amounts of dye sorbed somewhat lower, than those that would have been reached at the respective temperatures had sufficient time been allowed for equilibrium to be reached.

TABLE VI

SORPTION OF DYE BEFORE AND AFTER RAPID COOLING

Acid	Temperature, °C.	pH	C_B	C_F
0.0077 *M* HCl	100°	2.27	0.0314	0.215
"	70°	2.25	0.0013	0.216
0.0038 *M* H_2SO_4	100°	3.52	0.800	0.388
"	80°	—	0.605	0.400
0.0077 *M* H_2SO_4	100°	2.55	0.314	0.416
"	80°	—	0.201	0.423
0.0037 *M* H_2SO_4	100°	3.96	2.321	0.446
"	80°	4.23	2.182	0.455
"	60°	4.37	2.055	0.462

TABLE VII

SORPTION OF DYE AT ROOM TEMPERATURE AFTER A PRELIMINARY BOILING AND REMOVAL OF WOOL

Strength of sulphuric acid 0.0040 *M*

Time	C_B	C_F	pH	pH blank*
After first removal at 100°C.	0.996	0.407	3.48	2.94
After immersion of 5 min.	0.784	0.417	3.59	3.01
" " " 1 hr.	0.632	0.426	3.92	3.02
" " " 18 hr.	0.202	0.451	4.15	3.02

**Bath without dye.*

DISCUSSION

The sorption data in Table II and the corresponding curves of Fig. 1 show definitely that as the temperature is lowered, the amount of Orange II dye sorbed by the wool becomes greater. The effect is quite large and the reason why it does not seem to have been noticed previously is that most investigators have been satisfied with the determination of the dye on the fiber without determining the change in dye bath concentration. Under the conditions of our ex-

periments and those of most of the previous investigations on acid dye sorption, the exhaustion of the dye bath is almost complete. It follows that there can be an appreciable change in the concentration of the dye in the bath without a significant change in the amount of dye on the fiber. Thus if 99% of the dye originally in the bath is sorbed in one case, and 99.5% in another, the change might be considered very small, but the resulting dye bath concentration in the

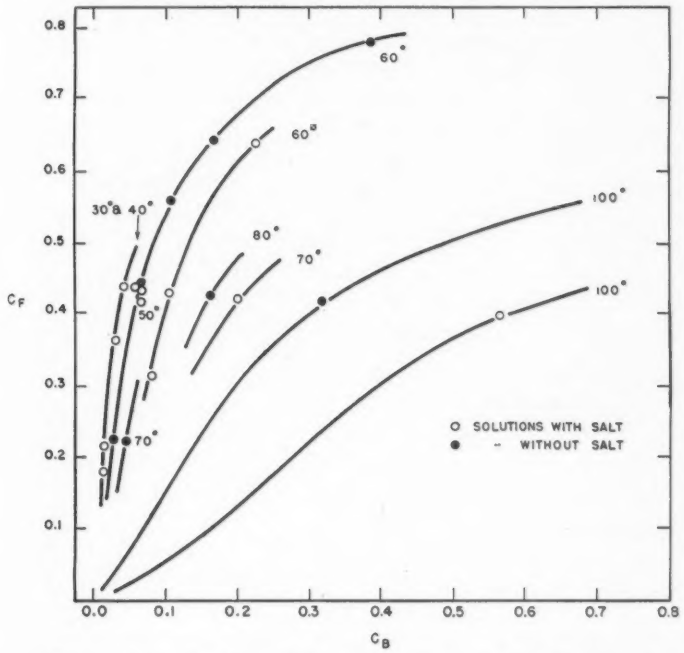

FIG. 1. Amount of Orange II sorbed by wool at various temperatures.
C_F = millimoles of dye sorbed per gram of wool.
C_B = millimoles of dye per liter of solution.

first case is twice that in the second and the conditions represent two widely differing equilibria. The determination of exact equilibrium conditions in such experiments is hampered by what appears to be a slow decomposition of the wool by the acid and catalyzed by the dye anions. This effect has been noted previously by a number of investigators, but in solutions more acid than our own (6, 7, 8, 11).

We have also noticed the change of pH with time that Lemin and Vickerstaff noticed in their experiments with the free acid of Naphthalene Orange (8). Table III shows the change obtained with two different strengths of acid in the presence of Orange II while Table IV shows the change in a sulphuric acid solution without dye. In the latter, the change was not noticeable at room temperature but it was marked at 95°C. This change in acidity is probably due,

as the authors have indicated, to a partial neutralization of the acid by ammonia set free as the result of wool decomposition. There is also a certain buffering action since the change in pH does not correspond to that in acid concentration determined by titration and given under C_B in Table IV. The change in acidity with time, except for a small corresponding change in the maximum dye absorptive capacity of the wool which results from it, does not appear to affect appreciably the dye sorption equilibrium, as shown by the data in Table I. This is further corroborated by the agreement between the results under *A* and *B* in Table II for the experiments carried out at 60° and 70°C. in acid solution without sodium sulphate. The preliminary boiling of the bath in *B* would be expected to have produced more decomposition than in *A*, yet the same equilibrium conditions were obtained in the two series of tests. It would seem then that the differences between the *A* and *B* results obtained with sodium sulphate in the bath are associated in some way with the presence of this salt. However, the differences are so small that they are not reflected in the C_F values and when the equilibrium curves are plotted the differences can be neglected in comparison with the differences between the various sorption isomers.

All the values of C_F and C_B were calculated on the original dry weight of the wool and are in error by an amount corresponding to the percentage loss in weight of the wool during the test. Attempts were made to determine this loss in a number of experiments after the wool had been dyed at the boiling point for one and one-half hours and in no case was the loss found to be more than 1%. This is actually a maximum since the dry weight of the wool after drying was obtained from the total dry weight by subtracting the weight of the calculated amount of dye on the fiber and the latter was somewhat too large by an amount corresponding to the dye lost in rinsing the wool before drying it.

Although the time necessary to reach equilibrium in the dye bath is greater the lower the temperature, the temperature effect on the equilibrium is so great that a marked change takes place in a comparatively short time as indicated by the results given in Tables V, VI, and VII. Owing to the possible wool decomposition to which reference has already been made, it is difficult to determine exactly when equilibrium conditions are reached. Examination of Table I shows that at 30°C., equilibrium has apparently been attained after the third day since little change occurred from then on to the 5th day, but prolonged tests carried to the 12th day indicated a gradual decrease in dye bath concentration with time. At 60°–70°C. two days were apparently sufficient to obtain equilibrium.

Table V shows that when a solution with an equilibrium dye concentration of 0.30 milliequivalents per liter at 100°C. was allowed to cool slowly, the dye concentration changed rapidly and in 42 min., at which time the temperature had reached 55°C., it was only one-fifth of its value at 100°C. It should be noted that this change was accompanied by a small but probably significant change in pH. This could hardly be ascribed to decomposition of the wool as the time was very short. It is our view that this change is due to an increase in acids sorbed by the wool. Other experiments carried out in our laboratories and which are to be reported later indicate that, as the quantity of dye sorbed increases, the amount of total acid sorbed also increases.

The results of Table VI, which were obtained after a rapid cooling of the dye solution from the boiling point to the temperatures given, also bring out the pronounced effect of temperature. It is obvious from these results that, if one wishes to study equilibrium conditions at the boiling point or at any other fairly high temperature, sampling has to be done at the temperature in question, otherwise any change in the temperature of the bath before sampling will disturb the equilibrium with consequent changes in dye and acid concentrations. The data in Table VII emphasize the previous remarks. They show the rapid change that takes place during the initial stages of the sorption at the lower temperatures and the change in pH of the solution as the dye is sorbed. This change in pH does not occur when no dye is present.

At this stage of the investigation it was thought that the increase in sorption as the solution was cooled may have been the result of some adsorption on the surface of the fibers. In order to determine whether this effect could explain the changes noted, the sorption was measured under similar conditions with two different wools, a fine wool having a mean fiber diameter of 21.3 μ and a coarser wool with a mean fiber diameter of 36.3 μ. Equal weights of these two wools have a surface ratio of 1.7 and should show a difference in sorption values if the effect in question is one of surface adsorption.

The results obtained with the two wools when the solution was cooled rapidly did show some difference in behavior, the fine wool showing a greater change on cooling than did the coarse wool. However, as these results were not conclusive, since the differences noted could have been due simply to a difference in the rate with which equilibrium was established in the two wools, the tests were repeated under conditions which would allow equilibrium to be reached. The results are given in Table VIII. Individual test results are reported to show what degree of agreement was obtained in our sorption experiments. These results

TABLE VIII

SORPTION OF DYE AT 60°C. BY A FINE WOOL AND A COARSE WOOL

H_2SO_4 concentration, 0.00745 M; initial pH, 1.90; time of immersion, 48 hr.

Wool quality	C_F	C_B	pH
Fine	0.748	0.242	2.27
	0.749	0.236	2.27
	0.749	0.250	2.27
Coarse	0.750	0.218	2.28
	0.750	0.236	2.28
	0.750	0.238	2.27
Fine	0.402	0.0436	2.19
	0.401	0.0440	2.19
	0.401	0.0440	2.18
Coarse	0.401	0.0452	2.18
	0.401	0.0444	2.19
	0.400	0.0440	2.19

show definitely that the equilibrium values for the fine wool are the same as for the coarse wool. The increased sorption taking place when the dye solution is cooled is evidently not the result of adsorption on the surface of the fibers.

The results obtained at 60°C. without salt in the bath were plotted in the same way as were our previous results at 100°C. (1), namely, by using the relation

$$\frac{C_B^{\,n}}{C_F} = \alpha + \beta C_B^{\,n}.$$

In this way n was found to be 1.22 and the maximum combining capacity of the wool, given by $1/\beta$, was 0.856 milliequivalent per gram. The corresponding values obtained at 100°C. for a similar strength of acid were 1.55 and 0.675, respectively.

The heat of sorption and change in free energy during sorption may be calculated from our results, but the values obtained will depend on the mechanism assumed for the process. If we apply the Gilbert and Rideal relations (5), as Lemin and Vickerstaff have done, to calculate the affinities of some dye acids for wool at 60°C. (8), we may use the following relation.

$$-(2\Delta\mu_D - \Delta\mu_{SO_4}) = RT \ln \frac{\theta_D^2}{\theta_{SO_4}} - RT \ln \frac{[D]^2}{[SO_4]} - RT \ln (1 - \theta_D - \theta_{SO_4})$$

Where $\Delta\mu_D$ is the difference between the chemical potential of the dye anion in the fiber and that in the solution, $\Delta\mu_{SO_4}$ is the corresponding difference for the sulphate ion, θ is the fraction of available sites on the fiber occupied by the ion indicated by the subscript, while $[D]$ and $[SO_4]$ are the concentrations of the dye anion and sulphate ion respectively in the solution. The above relation assumes that each sulphate ion occupies one site only. However we have found that our results agree better with the relation

$$-(2\Delta\mu_D - \Delta\mu_{SO_4}) = RT \ln \frac{\theta_D}{\theta_{SO_4}} - RT \ln \frac{[D]^2}{[SO_4]},$$

which is obtained if we regard the sulphate ion as occupying two sites on the fiber. In other words when one sulphate ion on the fiber is replaced by two monovalent dye anions, there is no change in the number of sites occupied and the equilibrium can then be represented as

$$W{:}\,SO_4 + 2D \rightleftharpoons WD_2 + SO_4,$$

from which we can write

$$\frac{W D_2}{W SO_4} = K\frac{[D]^2}{[SO_4]} = \frac{\theta_D}{\theta_{SO_4}}.$$

The above relations lead to values of -4.81 kcal. for $\Delta\mu_D$ at 100°C. and -4.97 kcal. at 60°C., if we take $\Delta\mu_{SO_4} = -0.90$ kcal. (13, p. 337). These values give $\Delta H = -5.93$ kcal. and $\Delta S = -4.2$ cal. per degree. The affinity values are of the same order as those given by Vickerstaff (14, p. 343) but the heat of sorption is only half that estimated from the data of Steinhardt,

Fugitt, and Harris for the interval 25°–50°C. (13). However, the value calculated from the latter data depends on what one assumes for the maximum combining capacity of the wool for Orange II. With an assumed value of 0.90 milliequivalent per gram or higher the calculated value for the heat of sorption is less.

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THE ESTIMATION OF VITAMIN A IN LOW POTENCY OILS[1]

By D. H. Laughland[2]

ABSTRACT

The use of the Morton–Stubbs correction in the analysis of low potency vitamin A containing oils may give rise to certain difficulties unless the limitations of the method are understood. Some of the theoretical and practical aspects of the problem have been studied with a view toward the clarification of difficulties inherent in the estimation of vitamin A. The nature of the absorption curve for the whole oil and nonsaponifiable fraction has been investigated and the influence of some manipulative errors on the estimation of potency has been determined. The results of a collaborative assay have been reported to provide information regarding interlaboratory differences. The necessity for extreme care in estimating the absorbancy of oil solutions and the importance of correct calibration of spectrophotometric equipment have been emphasized.

INTRODUCTION

The spectrophotometric examination of organic solvent solutions of vitamin A containing oils is the most popular method for the estimation of the vitamin. However, irrelevant absorption can interfere markedly in the application of such a procedure to low potency oils containing 1000–3000 I.U. vitamin A per gm. During the past few years various correction procedures have been designed which may be applied to spectrophotometric data to adjust for the influence of irrelevant absorption. The correction which is now official in the U.S.P. (9) and the Methods of Analysis, Association of Official Agricultural Chemists (8) is modified from the original work of Morton and Stubbs (6). Certain limitations, however, have been imposed by the adoption of correction procedures and it is necessary that they be understood.

The Subcommittee on Fat-soluble Vitamins of the World Health Organization of the United Nations (10) recommended in April, 1949, that the International Unit be defined as the activity of 0.344 μgm. of the standard preparation of crystalline vitamin A acetate which is equivalent to 0.3 μgm. of vitamin A alcohol. The absorption coefficient of vitamin A alcohol in isopropanol solution was stated to be 1750 and the conversion factor 1900. The committee also stated that the conversion factor of 1900 was applicable under the following conditions: (1) that the absorption maximum shall be within the range 325–328 mμ. (2) that the shape of the absorption curve shall agree closely with that of the international standard measured under the same conditions and compensated with a solution of the diluent oil. Intensities of absorption in the region 310–350 mμ expressed as decimal fractions of the maximum should not differ between sample and standard by more than 0.02; the absorption curves for vitamin A alcohol and acetate, expressed in the above manner are given by Morton and Stubbs (7).

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Absorption curves failing to meet the above requirements may be corrected to allow for irrelevant absorption, provided that the maximum is not displaced in wave length, by a geometric procedure (6).

The purpose of the work described in this paper was to attempt to assess the limitations of the correction when applied to low potency oils.

EXPERIMENTAL

The oils used in this study were samples of low potency oils which were offered for sale by various commercial firms and submitted by government inspectors to this laboratory for analysis. They ranged in potency from 800–3000 I.U. per gram. The saponification procedure was that outlined in the Methods of Analysis A.O.A.C. (8) and specially purified solvents were used throughout. Skellysolve B was shaken with concentrated sulphuric acid until no further color change was observed in the acid. The Skellysolve was then washed with dilute sodium hydroxide and water and distilled. Isopropanol was redistilled before use. Absorption measurements were made with a Model DU Beckman spectrophotometer employing silica cells and a hydrogen discharge source. Oil samples were stored in a refrigerator and absorption measurements were made as soon as possible after preparation of the sample. In the event that an oil residue was transferred from one solvent to another the evaporation of solvent was carried out in an atmosphere of nitrogen.

THEORETICAL CONSIDERATIONS

The Nature of the Irrelevant Absorption in Low Potency Feeding Oils

The linearity of the irrelevant absorption in an oil sample which is removed by saponification may be observed from the "difference-curve", which is obtained by subtracting the absorbancy values for the nonsaponifiable fraction from corresponding values for the whole oil. The "difference curves" are generally nonlinear over the region 310–334 mμ, yet it is usually found that linearity exists if the absorbancies at 310, 325, and 334 mμ alone are considered.

In the application of the Morton–Stubbs correction it is not essential that irrelevant absorption be linear over the entire region 310–334 mμ provided the absorbancies at the three wave lengths used in the determination be on the same line. It should be pointed out that there may be little justification for assuming that the "difference curve" represents the character of the irrelevant absorbing material remaining in the sample after saponification. The Morton–Stubbs procedure corrects for the irrelevant absorption remaining in the sample after saponification and this may differ markedly in spectral characteristics from that which is susceptible to saponification.

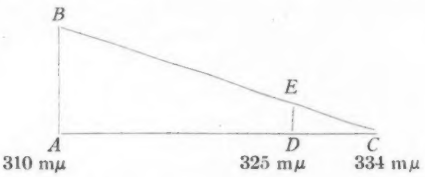
The method to be described is a simple procedure which permits the calculation of a theoretical value from which the deviation of irrelevant absorption from linearity may be computed for the "difference curve". This is possible when the absorbancy values at 310, 325, and 334 mμ are known for the whole oil and nonsaponifiable fractions.

If irrelevant absorption is assumed to be linear the value of the absorbancy at 325 mμ for the "difference curve" will be determined by the following relationship:

$$(1) \qquad A_{325\ m\mu} = A_{334\ m\mu} + 0.375\ (A_{310\ m\mu} - A_{334\ m\mu})$$

where $A_{325m\mu}$, $A_{334m\mu}$, $A_{310m\mu}$ = irrelevant absorption at 325, 334, and 310 mμ respectively. The irrelevant absorption at any wave length is the difference between the absorption values for the whole oil and nonsaponifiable fractions.

This equation is based on the assumption, which is usually true for low potency oils, that irrelevant absorption is greater at 310 mμ than at 334 mμ. In the event that the reverse condition is encountered suitable adjustments may be made in the expression. The irrelevant material remaining in the nonsaponifiable fraction often possesses greater absorption at 334 mμ than at 310 mμ as indicated in Table III. The factor 0.375 is derived from the expression used to calculate the irrelevant absorption at any point intermediate between two wave lengths, i.e.,



$$\frac{DE}{AB} = \frac{(334 - 325)}{(334 - 310)} = \frac{9}{24} = 0.375.$$

Therefore $DE = 0.375\ AB$.

Equation (1) has been applied to the absorption data obtained in the analysis of 17 samples of low potency oil and the results are shown in Table I. Four of these samples have absorbancy values at 325 mμ which differ from the theoretical by more than 5%. This indicates that the irrelevant absorbing material removed by saponification is nonlinear where the absorbancy at 310, 325, and 334 mμ is considered.

In the event that it is possible to demonstrate linearity in the absorption of the irrelevant material removed by saponification it is possible to make certain calculations regarding the total irrelevant absorption in a sample by the application of the Morton–Stubbs equation to the absorption data for the whole oil fraction (2). Saponification is relatively ineffective in removing irrelevant absorption from some oil samples and a chromatographic technique may often be used to advantage in characterizing the irrelevant material (1, 3, 5, 4, 2).

The extent to which saponification reduced the extinction coefficient at 325 mμ was calculated for 100 oil samples. The data in Table II indicate the frequency (in per cent) of occurrence of the whole oil/nonsaponifiable ratios between arbitrary limits.

TABLE I

THEORETICAL ABSORBANCY AT 325 mμ FOR "DIFFERENCE CURVE"*

Sample	Extinction coefficient at 325 mμ		Irrelevant absorption ext. coeff. at 325 mμ		
	W.O.	N.S.	Obs.	Calc.	% Diff.
1	0.992	0.772	0.220	0.228	3.50
2	0.713	0.529	0.184	0.185	0.54
3	1.333	0.591	0.742	0.726	0.22
4	0.822	0.591	0.231	0.227	1.76
5	1.616	1.320	0.296	0.286	3.50
6	1.249	0.815	0.434	0.425	2.12
7	1.375	0.723	0.652	0.663	1.67
8	0.855	0.652	0.203	0.194	4.64
9	0.861	0.729	0.132	0.152	13.18
10	1.031	0.812	0.219	0.216	1.39
11	0.923	0.652	0.271	0.276	1.81
12	1.604	1.355	0.249	0.288	13.52
13	0.593	0.336	0.257	0.253	1.58
14	1.446	1.120	0.326	0.294	10.89
15	0.721	0.555	0.166	0.175	5.14
16	0.835	0.633	0.202	0.211	4.26
17	1.116	0.802	0.314	0.304	3.29

**Each value is the mean of two determinations carried out with separate weighings of the oil sample in isopropanol solution. The whole oil fraction is designated W.O. and the nonsaponifiable fraction is N.S.*

TABLE II

DISTRIBUTION OF OILS WITH REGARD TO EXTINCTION RATIO AT 325 mμ

$A_{325m\mu}$ (W.O.)/$A_{325m\mu}$ (N.S.)	Per cent of oils
1.00 – 1.05	4
1.05 – 1.10	7
1.10 – 1.15	14
1.15 – 1.20	11
1.20 – 1.25	13
1.25 – 1.30	18
1.30 – 1.35	9
1.35 – 1.40	11
1.40 – 1.45	7
1.45 – 1.50	6

The results indicate that 56% of the oils had an absorption ratio which was between 1.10 and 1.30 and 76% had a ratio between 1.10 and 1.40. The extinction coefficient at 325 mμ is about 15-20% below that of the whole oil fraction in 50% of the low potency oils analyzed in this laboratory. In the experience of this laboratory it has been found that the Morton–Stubbs correction reduces the value of the extinction coefficient of the nonsaponifiable fraction of a low potency oil by an additional 15-25% in most cases.

The Value of the Absorption Ratio after Saponification

One of the criteria stated previously for the suitability of absorption data in order to apply a conversion factor of 1900 was that absorption ratios for

wave lengths between 310 and 334 mμ should not differ by more than 0.02 from the corresponding values for the Reference Standard. That this is seldom found for the nonsaponifiable fraction of low potency oils is shown in Table III. There is some evidence that the greatest divergence from the allowable value is found with oils in which a marked reduction in the value of the extinction coefficient occurs on saponification. The data in Table III have been arranged to demonstrate that there is a tendency for the absorption ratio at 310 mμ to deviate from

TABLE III

ABSORPTION RATIOS FOR NONSAPONIFIABLE FRACTION IN ISOPROPANOL

Sample	Absorption ratio, 310 mμ	Ext. coeff. (325 mμ)		N.S. as % of W.O.
		W.O.	N.S.	
Ref. Std.	0.857	5.37	5.28	98.3
1	0.856	0.868	0.802	92.3
2	0.858	1.546	1.420	91.8
3	0.859	1.616	1.342	83.0
4	0.875	0.712	0.555	77.9
5	0.880	1.447	1.120	77.4
6	0.875	0.822	0.625	76.0
7	0.881	0.835	0.633	75.8
8	0.886	0.733	0.529	72.3
9	0.890	1.249	0.797	63.8
10	0.890	1.116	0.672	60.2
11	0.970	0.597	0.360	60.3
12	0.903	1.370	0.723	52.7

the theoretical value of 0.857 as the amount of irrelevant absorption in the sample increases. The extinction coefficient of the nonsaponifiable fraction has been expressed as a percentage of the whole oil extinction coefficient and the amount of irrelevant material removed by saponification is inversely related to this percentage. The usual saponification procedure appears to be incapable of removing all the irrelevant material from these heavily contaminated oils and it is therefore necessary to use the Morton–Stubbs correction to compensate for the residual irrelevant absorption. Chromatography appears to offer a reasonable technique by which much of the irrelevant absorbing material may be removed and there is some evidence (2) that such a technique removes more irrelevant material than saponification.

Influence of an Error in Measuring the Absorbancy of an Oil Solution

It is necessary to attain a high degree of precision in estimating the absorbancy of an oil sample if a correction is to be applied. The influence of an assumed error in measuring the absorbancy may be seen from the examples which have been worked out using the following values for the nonsaponifiable fraction of the Reference Standard.

$E^{1\%}_{1cm.}$, 325 mμ (isopropanol) = 5.26.

Fixation points 310 and 334 mμ.

Absorbancy at fixation points = 4.509, i.e., absorbancy at these points is 6/7 that observed at the maximum (325 mμ) and the absorption ratio is therefore 0.857.

Conversion factor 1900.

Example 1

The calculation of potency without application of a correction.

Assume that an error of + 5% is made in estimating the absorbancy of the standard.

The observed extinction coefficient is:

$$5.26 \times \frac{105}{100} = 5.523.$$

The calculated potency is 5.523 × 1900 = 10,494 I.U. per gm.

The estimated potency is 4.94% too high when this procedure is used. It is not exactly 5% too high because the conversion factor of 1900 is an approximate figure. In exact terms it should be

$$\frac{10{,}000}{5.26} = 1901.1.$$

In the example cited above the calculated potency is in error by an amount which is equivalent to the error of the absorbancy measurement.

Example 2

The calculation of potency when a correction is applied.

If an error of + 5% in the absorption measurement at 325 mμ is assumed and the values previously cited for the standard are used the following values are obtained.

$$A_{325\ m\mu} = 5.523,$$

$$A_{310\ m\mu} = 4.509,$$

$$A_{334\ m\mu} = 4.509.$$

By substitution in the equation given in the A.O.A.C. Methods of Analysis (8),

$$A(\text{corrected})\ 325\ m\mu = 7\ A_{325\ m\mu} - 2.625\ A_{310\ m\mu} - 4.375\ A_{334\ m\mu},$$

$$= 38.661 - 11.836 - 19.727,$$

$$= 7.098.$$

The calculated potency is 7.098 × 1900 = 13,486 I.U. per gm. which is 34.86% too high.

In the application of the correction an error in estimating the absorbancy is magnified in the final result. It is unlikely in practice that an error will be made in estimating the absorbancy at only the wave length maximum and that the correct values will be observed at the fixation points. It is more likely that an error made in one reading will be modified by a similar error in another reading. The necessity for accurate absorption measurements is apparent.

In Table IV, the influence of various errors on the extinction coefficient of the Reference Standard has been calculated. It was assumed in making these calculations that an error was made in estimating the absorbancy at one wave length but that the theoretically correct values prevailed at the other two critical wave lengths in a fashion similar to the example cited previously.

TABLE IV

THE INFLUENCE OF ASSUMED ERRORS IN ABSORBANCY MEASUREMENTS ON THE VALUE OF THE EXTINCTION COEFFICIENT OF THE REFERENCE STANDARD

Error	$A_{325m\mu}$ (corrected)	% Error
+ 2% 325 mμ	1.141	+ 14.1
+ 5% " "	1.351	+ 35.1
+ 2% 310 mμ	0.956	− 4.4
+ 5% " "	0.888	− 11.2
+ 2% 334 mμ	0.927	− 7.3
+ 5% " "	0.813	− 18.7

The Influence of a Calibration Error on the Corrected Extinction Coefficient

It may be demonstrated by taking absorbancy measurements at 1 mμ intervals over the regions surrounding the critical wave lengths used in the Morton–Stubbs correction that an almost constant difference exists between successive readings. In terms of the absorption ratio this amounts to about 0.020 on the absorbancy scale at 310 and 334 mμ and 0.006 at 325 mμ.

These values have been used to calculate the apparent absorbancy which would be evident for various errors in calibration of the spectrophotometer.

The term "low calibration" is used to describe the case in which the true wave length is higher than that indicated by the wave length dial, i.e., 325 (obs.) was 326 (actual). A "high calibration" means the converse. The assumption is made, which is not unreasonable in working over a narrow wave length region, that a calibration error is reflected to the same extent at all wave lengths. The apparent absorption ratios under the conditions outlined above are recorded in Table V for the nonsaponifiable fraction of the Reference Standard in isopro-

TABLE V

APPARENT ABSORBANCIES WITH AN ASSUMED CALIBRATION ERROR

Calibration error, mμ	Apparent absorbancy			Corrected $A_{325m\mu}$
	310 mμ	325 mμ	334 mμ	
No error	0.857	1.000	0.857	1.001
High 0.5	0.847	0.997	0.867	0.963
1.0	0.837	0.994	0.877	0.924
1.5	0.827	0.991	0.887	0.886
Low 0.5	0.867	0.997	0.847	0.998
1.0	0.877	0.994	0.837	0.994
1.5	0.887	0.991	0.827	0.991

panol solution. The theoretical value of 0.857 at 310 and 334 mμ represents 6/7 the absorption at 325 mμ in accordance with the requirements of the Morton–Stubbs correction.

It will be observed that the corrected absorbancy deviates from the theoretical to a greater extent when there is a "high calibration" error than when there is a "low calibration" error. In the case where the spectrophotometer is calibrated 1.0 mμ too high the corrected absorbency is 7.6% too low, whereas the corrected absorbency is only 0.6% too low when the instrument is calibrated 1.0 mμ too low.

It is of course possible to use these data to test for the presence of a calibration error. If, for example, a near-constant error is encountered in the application of a correction procedure to the Reference Standard the wave length calibration of the instrument should be checked. The ratio $A_{334m\mu}/A_{310m\mu}$ has been calculated for the data in Table V and the results are shown graphically in Fig. 1. It is

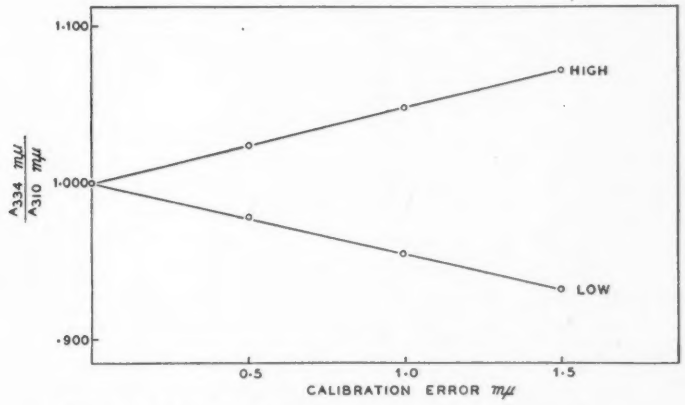


FIG. 1. Influence of a calibration error on the ratio $A_{334m\mu}/A_{310m\mu}$.

possible, by using these graphs, to determine both the direction and magnitude of a calibration error. The necessity for frequent and careful calibration of spectrophotometric equipment is obvious.

Other Aspects of Irrelevant Absorption

It is often advantageous in studies related to the theoretical aspects of irrelevant interference to express absorbancies in terms of the "absorption ratio" between two wave lengths. This is done by assigning a value of unity to the absorbancy at one wave length and then calculating the corresponding values for other wave lengths. The wave length at which maximum absorption occurs may be conveniently selected as the reference point. If this is done with the nonsaponifiable fraction of the Reference Standard in isopropanol solution, for example, the absorption ratio at 310 mμ and 334 mμ is 0.857. Absorption ratios are of considerable worth in assessing the atypical nature of an observed curve.

It is possible, assuming that irrelevant interference is linear, to use these curves to ascertain the slope of irrelevant absorption by the method to be described. Calculations have been made to show the influence of linear irrelevant absorption on the shape of the absorption curve of the Reference Standard and the results are given in Table VI. The hypothetical linear interference was assumed to have an absorbancy value of 0.100 at 340 mμ in each case and to exhibit the slope indicated in the table. An increase in the slope of irrelevant absorption

TABLE VI

INFLUENCE OF LINEAR EXTRANEOUS ABSORPTION ON POSITION OF MAXIMUM

Wave length (mμ)	Ref. Std. (N.S.)	Calculated absorption with assumed slope			
		0.25	0.50	0.75	1.00
300	0.623	0.823	0.923	1.023	1.123
305	0.735	0.923	1.010	1.098	1.185
310	0.860	1.035	1.110	1.185	1.260
315	0.910	1.073	1.135	1.198	1.260
320	0.965	1.115	1.165	1.215	1.265
325	1.000	1.138	1.175	1.213	1.250
330	0.939	1.064	1.089	1.114	1.139
340	0.719	0.819	0.819	0.819	0.819

results in a shift in the absorption maximum to lower wave lengths and this condition is encountered in samples of low potency oils which exhibit maxima in the region 315-320 mμ. This indicates that the irrelevant contribution to the observed absorbancy in the curves for the whole oil fraction is greatest at wave lengths below 325 and that the slope of irrelevant linear absorption may approach a value of 1.0. The absorption ratios at three wave lengths have been plotted against slope in Fig. 2.

It is possible, assuming that irrelevant interference is linear, to ascertain the slope of the line which represents irrelevant absorption by the use of these curves. The usefulness of this device is limited, however, since the value of the absorp-

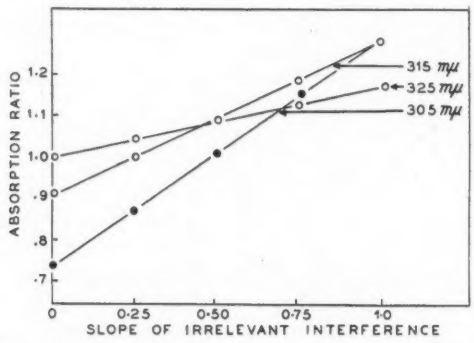


FIG. 2. The use of absorption ratios in determining the slope of irrelevant absorption.

tion ratio is dependent upon the position of the line representing irrelevant absorption above the abscissa. In practice the lines representing irrelevant absorption would not all intersect at 340 mμ as indicated by the data in Table VI.

It should not be assumed that all the irrelevant absorption with which it is necessary to deal in the analysis of low potency oils is found in the oil sample.

It is the exception rather than the rule to find commercial solvents which are suitable for precise spectrophotometric measurements without prior purification. Commercial Skellysolve B which has many desirable properties as a solvent for spectrophotometric measurements should be treated with concentrated sulphuric acid then washed with dilute alkali and water before distillation. This treatment removes a considerable amount of material which interferes in absorption measurements.

Certain solvents such as acetone may interfere markedly in absorption measurements unless special precautions are taken when vitamin A is transferred from acetone solutions to other solvents. It is common practice to evaporate aliquots of an organic solvent solution by placing the tube in a water bath and blowing a stream of nitrogen over the solvent. Unless the tube is heated for a minute or two after the residue appears dry and the tube thoroughly flushed with nitrogen, a small amount of acetone vapor may be left in the tube. Condensation of this vapor on cooling the tube may result in erroneous absorption measurements. It has been found for example, that 0.25% acetone in Skelly B has an absorbancy value of 0.226 at 300 mμ and 0.015 at 325 mμ when read against a Skelly blank.

It is necessary to make certain that the cuvettes employed in making the absorbancy measurements are properly standardized. The matched cuvettes supplied with the spectrophotometer are usually suitable for vitamin A estimations. However, care should be exercised when replacements are obtained to make certain that the transmittancy of the cuvette is satisfactory and corresponds to that of the other cells in the set.

Care should be exercised in cleaning cuvettes to assure the absence of any film on the cell surface which can affect the transmittancy. It is probably worthwhile, after rinsing the cuvettes, to store them submerged in solvent at all times when they are not in use. In spite of this precaution a film may collect on the cell surface and impair the transmittancy. In the experience of this laboratory it has been found necessary to treat the cells with warm alcohol from time to time in order to preserve the transmittancy. In some cases, treatment with dilute acid has been found effective.

COLLABORATIVE ASSAY RESULTS

During the past year this laboratory and several others routinely concerned with the analysis of vitamin A in low potency oils participated in a collaborative assay. Samples of oil were prepared in this laboratory and four lots, each consisting of three samples of oil, were submitted to each participating laboratory. No suggestions regarding the procedure to be followed in the analysis were made since it was desired to obtain an estimate of potency under the conditions

TABLE VII

RESULTS OF QUESTIONNAIRE SUBMITTED TO OIL FIRMS IN 1950

Firm No.	Frequency of spectrophotometer calibration	Frequency of check analyses using Ref. Stand.	Are analyses done on whole oil or nonsap.	In what units are results reported	Do you apply a corr. to absorption data	Have you ever encountered an increase in ext. coeff. after saponification
1	Every three months	Every three months	Nonsap.	"Spec" units ($E \times 2000$) Int. units	No	No
2	Daily	Monthly	Nonsap. below 10,000 I.U./gm.		No	No
6	Yearly	Std. run with unknown	occasionally on nonsap.	U.S.P. units	No	No
8	Irregular intervals	No set frequency	Nonsap. below 10,000 I.U./gm	Bio-units	No	Yes
9	Not stated	Std. run with unknown	Nonsap.	Int. units	No	No
11	Occasionally	Every few months	Nonsap. below 10,000 I.U./gm.	Int. units	Occasionally	No
10	Not stated	Every second week	Depends on circumstances	Spectrophotometric units	No	Rarely
3	Every two years	Routinely	Nonsap.	U.S.P. units (2000 factor)	Yes	Yes in some dogfish liver oils
7	Occasionally	Every three months	Either	U.S.P. or I.U. (2000 factor)	No	No
12	Monthly	Monthly	Nonsap.	Int. Units	No	No

currently employed. A questionnaire was sent to the firms prior to the initiation of the assay in an attempt to gain some information regarding the general procedure followed in the various laboratories. The replies to some of these questions are recorded in Table VII.

In view of the difficulties inherent in the use of a correction procedure it is necessary to do everything possible in order to minimize experimental errors.

1. The wave length scale of the spectrophotometer should be calibrated at monthly intervals. Calibration should be carried out more often if the instrument is subjected to appreciable vibration.

2. It is necessary to pay careful attention to detail in carrying out the analytical procedure. Particular care should be exercised in the purification of solvents and in transferring and evaporating aliquots of the extract containing the vitamin.

3. In laboratories routinely concerned with the estimation of vitamin A the Reference Standard should be analyzed at least once a week.

4. Laboratories employing the correction for the first time should carry out duplicate or triplicate determinations on all samples until a degree of familiarity has been attained. If at all possible, duplicate determinations should be carried out routinely in all laboratories because errors in estimating the absorbancy are magnified in the application of the correction.

The procedure outlined in the Methods of Analysis, Association of Official Agricultural Chemists (8) became official as regards regulations of the Feeding Stuffs Act in Canada on Sept. 1, 1951, and this should obviate many of the discrepancies which have appeared heretofore in the analytical procedures employed in various laboratories.

The incomplete replies received for the first lot of oils sent out made it impossible to analyze the results statistically. However, six firms submitted complete data for the succeeding three lots of oils and the coefficients of variability for the extinction coefficients of the samples are shown in Table VIII.

TABLE VIII

COEFFICIENTS OF VARIABILITY OF EXTINCTION COEFFICIENTS IN COLLABORATIVE ASSAY

	Coefficient of variability					
	Lot 2		Lot 3		Lot 4	
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Sample 1	6.63	11.52	3.16	16.28	3.33	16.25
Sample 2	4.35	8.63	3.44	10.29	3.09	9.39
Sample 3	3.35	8.88	3.05	8.71	3.43	3.80

These results illustrate that the variability in the estimation of potency is markedly increased when a correction was applied to absorption data. Particular care must be exercised in estimating the absorbancy values and an attempt made to improve the precision of the method by refinements in technique.

The Influence of the Correction in the Routine Analysis of Low Potency Oils

It is not possible to define the influence of the correction in precise terms because of the variation in quality and quantity of irrelevant absorption in different samples. It has been the experience of this laboratory that the estimated potency is from 15-25% lower when a correction is used. Some indication of the influence of the correction may be seen in Table IX where the corrected, uncorrected, and antimony trichloride values are given for several oil samples.

TABLE IX

EFFECT OF CORRECTION ON THE ESTIMATED POTENCY OF LOW POTENCY OILS

Sample	Potency, I.U./gm.		
	Uncorrected	Corrected	$SbCl_3$
1	2389	1938	2228
2	2314	1965	2293
3	1617	1435	1611
4	1170	918	980
5	3000	2585	2660
6	2295	2079	2280
7	1199	932	1160
8	3640	3256	3360
9	2170	1850	2040
10	1370	1193	1310

It is generally found that the values obtained by the antimony trichloride test are intermediate between the uncorrected and corrected values.

The results obtained in 11 separate analyses using the nonsaponifiable fraction of the Reference Standard are given in Table X.

TABLE X

RESULTS OF ANALYSES OF REFERENCE STANDARD IN THE COLLABORATIVE ASSAY

	Uncorrected	Corrected
Mean $E^{1\%}_{1\text{ cm.}}$ 325 mμ	5.296	5.235
$E^{1\%}_{1\text{ cm.}}$ 325 × 1900	10,062	9,947
Standard deviation	0.044	0.090
Coeff. of variability	0.83	1.719

The mean absorption ratio of these samples at 310 mμ was 0.855 and at 334 mμ it was 0.862. The corrected value of the extinction coefficient at 325 mμ is about 1% below the uncorrected value.

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THE LACTIC STREPTOCOCCI IN EDMONTON MILKS AND CREAMS[1]

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ABSTRACT

Of 3000 isolates from 59 samples of commercial raw milks approximately 8% were typical lactic streptococci (*Streptococcus lactis* or *cremoris*), 2% were fecal streptococci, and 8.5% were alkali formers. Of the typical lactic streptococci 96% were *S. lactis* and 4% were *S. cremoris*. No typical lactic streptococci were isolated from over 50% of the milks while organisms of this group were found in 15% of the milks, in numbers exceeding 10,000 per ml. The incidence was higher in summer than in winter milks and there was inconclusive evidence that it followed the standard plate count. Two of the 18 winter milks did not sour normally. No lactic streptococci were isolated from 10 freshly HTST pasteurized milks and only one of these pasteurized milks soured normally under the predominating influence of typical *S. lactis*. It is probable that this milk was plant contaminated after pasteurization. No isolations of this group of bacteria were made from two udder milks or one foremilk, either before or after souring. Typical lactic streptococci predominated in 15 sour churning creams. In all sour samples in which typical lactic streptococci did not predominate, the majority of the bacteria were acid formers not typical of *S. lactis*. Fecal streptococci were isolated, usually in small numbers, from eight milks. It is concluded that *S. lactis* in its natural habitat does not flourish in this climate.

INTRODUCTION

Progress in Canadian dairy processing is impeded by meager knowledge of the simple, fundamental bacteriology of our dairy products. A northern climate may be expected to influence the indigenous bacterial flora and dairy processing routines should be expediently adjusted.

According to local folklore, milk did not turn sour when dairying was first introduced into this area. If the observation was correct, it confirms the statement of Hammer (1, p. 61) that "occasionally, in new dairy districts, souring of milk is markedly delayed; this probably is because the surroundings are less thoroughly seeded with lactic acid organisms than in the older districts so that the milk is but lightly contaminated".

There is a widespread belief among the cheesemakers of this area that it is more difficult here than elsewhere to make First Grade cheese from carelessly produced milk. An explanation is possible if *Streptococcus lactis* finds this an alien climate.

It is commonly accepted that, as stated by Russell and Hastings (3, p. 88), "Under ordinary farm conditions the larger part of '*S. lactis*' found in milk come directly from the utensils". During the 1930's the lactic acid bacteria, both coccus and rod forms, became increasingly difficult to isolate, by direct

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plating methods, from the raw milks of the Edmonton milkshed. Concurrently with this the milks were being progressively improved, largely by more rigorous bactericidal treatment of the milking utensils.

A study of the souring of milks in this area is, therefore, of importance, especially in view of the growing practice of infrequent delivery of raw milks to the pasteurizing plants, cheese factories, and condenseries, and of churning creams to the creameries.

METHODS

Samples

A total of 59 shippers' milks, sampled over a period of nine months, on arrival at Edmonton pasteurizing plants, were grouped seasonally. Ten pasteurized milks, 15 churning creams, and three carefully drawn udder milks including one foremilk were also examined. Plate counts were determined and isolations made after the 18 winter milks, the pasteurized milks, and the udder milks were soured at 25°C.

The quality of the raw milks is illustrated in Table I which shows that over half of the milks had standard plate counts of not over 50,000 per ml. No sample reduced methylene blue in less than 5 hr. while 22 samples did not reduce the dye in 10 hr.

TABLE I

THE QUALITY OF THE 59 RAW MILKS

Standard plate counts	Percentage classification			
	Spring milks	Summer milks	Winter milks	Total
< 10,000	25	9.5	5.6	13.6
10,001 – 50,000	30	57.1	27.8	39.1
50,001 – 100,000	15	14.3	33.3	20.4
> 100,000	30	19.0	33.3	27.2

Plating Methods

Standard plate counts on TGEM medium incubated at 35°C. for two days were determined for all samples. The 41 spring and summer raw milk samples were also plated on veal infusion glucose agar incubated at 25°C., for four days. All counts are reported on a 'per milliliter' basis.

Isolations

Areas, judged to be representative, comprising 30 colonies were marked on plates from suitable dilutions and all 30 colonies were fished into litmus milk. These litmus milk cultures were incubated at 35°C. for five days or 25°C. for seven days, depending on the incubation temperature of the plates from which they were isolated. Those cultures typifying *Streptococcus lactis* were then carried on Difco stock culture agar after incubation at the isolation temperature.

Definitions

When the litmus milk reaction, morphology, and Gram reaction were typical of *S. lactis* and no growth was observed at 45°C., the culture was considered to be a lactic streptococcus (called lactics in Table II). If, in addition, growth occurred at 45°C., the culture was considered to be a fecal streptococcus (called fecals in Table II).

For report purposes, all acid-forming cultures exhibiting atypical reactions in litmus milk are labeled other acid formers. Cultures effecting no change in litmus milk, except to turn it blue, were considered to be alkali formers. No change in the litmus milk indicated inert strains and transfer failures.

RESULTS

Lactic Streptococci

Choice of a Medium

Preliminary trials with raw milks and starters plated on seven media led to the choice of media as previously outlined. Results with the first 41 raw milks showed no average difference between the two techniques described above, although there was considerable, but not constant, disparity in the case of a few milks. In view of this, all further isolations were made from the Standard plates.

Incidence

Of a total of 3000 cultures, isolated from 59 commercial raw milks, 238 (7.9%) were grouped as lactic streptococci (Table II) but this percentage is misleading. No lactic streptococci were isolated from 32 (54.2%) of these milks, while lactic streptococci were found in numbers exceeding 10,000 per ml. in only nine of the milks.

Seasonal Variations

Table II would seem to indicate a higher incidence of lactic streptococci in spring than in summer milks as the former yielded twice the proportion of such isolations. This difference, however, is probably an artifact because nine of 20 spring milks and nine of 21 summer milks yielded no lactic streptococci. On the other hand, it is probable that the difference to be observed in the case of the winter milks represents an actual difference because no lactic streptococci were isolated from 14 of the 18 winter milks.

Relation to Total Count

The evidence is suggestive, but not conclusive, that the lactic streptococcus content varied with the total plate counts of the milks. This inconclusiveness was to be expected because of the quality of the milks studied. On the other hand, lactic streptococci accounted for 14.7% of the isolations from the 10 spring and summer milks having plate counts over 100,000, and only 3.7% of the isolations from the five spring and summer milks having plate counts under 10,000.

TABLE II

SUMMARY OF SAMPLES, ISOLATIONS, AND GROUPINGS

Samples		No. of isolates	Lactics		Fecals		Other acid formers		Alkali formers		Inert	
Kind	No.		No.	%	No.	%	No.	%	No.	%	No.	%
Spring milk	20	1200	137	11.4	38	3.2	731	61	74	6.1	220	18.3
Summer milk	21	1260	82	6.5	25	2	745	59	151	12	257	20.4
Winter milk	18	540	19	3.5	1	0.2	356	66	31	5.7	133	24.6
Total	59	3000	238	7.9	64	2.1	1832	61	256	8.5	610	20.3
Pasteurized milk	10	300	0	0	0	0	229	76.3	49	16.3	22	7.3
Udder milk	3	80	0	0	0	0	70	87.5	1	1.2	9	11.2
Churning cream	15	450	350	77.8	0	0	74	16.4	0	0	26	5.8
Sour winter milk	18	540	349	64.6	27	5	150	27.8	5	0.9	9	1.7
Sour pasteurized milk	10	300	107	35.7	0	0	174	58	6	2	13	4.3
Sour udder milk	3	80	0	0	0	0	48	60	0	0	32	40
Total	46	1370	806	58.8	27	1.9	446	32.5	11	0.8	80	5.8
Grand total	118	4750	1044	22	91	1.9	2577	54.3	317	6.7	721	15.1

Pasteurized Milks

Of the 10 pasteurized milks collected on different days, the first six were obtained at a union in the sanitary pipe line about nine feet from the exit of an HTST pasteurizer. The remaining four milks were collected at the bottler. No lactic streptococci were isolated from any of the 10 milks while the milks were still sweet.

Sour Milks and Creams

Of the 18 winter milks, 16 soured normally with lactic streptococci making up an average of 72.7% of the total flora and varying from 17% to 100% in the individual samples. No lactic streptococci were isolated from two of the sour milks, the souring was slow and other acid formers constituted almost 100% of the flora.

When the pasteurized milks were allowed to sour, no lactic streptococci were isolated from the first five samples. Milk number six, which also was taken from the pipeline, contained the normal number of lactic streptococci of sour milk, and this type constituted 90% of the total flora. It is probable that the pipeline was inadequately sterilized on that particular day. Only 7% of the flora of the seventh milk when it was soured were lactic streptococci, while 80% to 100% of the flora of the other three milks were lactic streptococci. Those sour pasteurized milks which lacked, or were low in, lactic streptococci were high in other acid formers.

Lactic streptococci made up from 50% to 97% of the flora of 15 churning creams which varied in titratable acidity from 0.27% to 0.63% expressed as lactic acid.

Udder Milks

No lactic streptococci were isolated from three udder milks, one of which was a foremilk, either before or after souring.

Fecal Streptococci

Fecal streptococci were isolated from two spring and five summer milks and one winter sample. When they were present, their numbers were, in general, low, but in two milks they were 27% and 37% respectively of the total isolations. They were found in considerable numbers, varying from 3.3% to 23% of the total isolates, in eight of the 18 soured winter milks. They were not isolated from 51 of the raw sweet milks, any of the pasteurized or udder milks, sweet or sour, or any of the churning creams.

Other Acid Formers

Other acid formers were found, usually in large numbers, in all samples except three churning creams, four sour winter milks, and one sour pasteurized milk. They were the major constituent of the flora of most of those sour milks and creams which were not soured by the action of lactic streptococci.

Study of this group was not carried further. Atypical strains of *S. lactis* were probably included and the group merits further investigation.

Alkali Formers

Alkali formers were encountered, sometimes in large numbers, in 47 of the sweet raw milks, in all but one of the pasteurized milks, and in one udder sample (not foremilk). This sample was probably contaminated during milking. They were not isolated from two of the udder milks or any of the churning creams.

Inert Cultures

A total of 721 (15%) of the 4750 isolates effected no observable change in litmus milk. These undoubtedly included both inert strains and transfer failures.

Identification

Harrison (2) identified 104 cultures chosen at random from the lactic and fecal streptococcus groups mentioned above. Ninety of these isolates were *S. lactis*, four *S. cremoris*, seven *S. liquefaciens*, and three *S. fecalis*. The identification of only one culture failed to confirm the original grouping as lactic or fecal streptococci.

DISCUSSION

Added to the numerous vagaries of plate counting, the isolation technique of this study undoubtedly introduced further inaccuracies of unknown magnitude. Their recognition should temper interpretation of the results.

Since 30 colonies per medium per sample were fished into litmus milk, it follows that on the average the specified group manifested its presence only when it constituted at least 1/30th of the total plate count. Thus, a lactic streptococcus count of zero did not always mean the absence of this group, as illustrated in the souring of the winter and pasteurized milks.

The identification of 104 cultures chosen at random proved the soundness of the groupings chosen and the accuracy of group placings, since only one of the 104 cultures had been improperly grouped. The lactic streptococcus group comprised 1044 cultures which, on the basis of the above identification, was composed of approximately 96% *S. lactis* and 4% *S. cremoris*.

These results support the belief that the climate of this area does not encourage *S. lactis* in its natural habitat; that this organism is introduced into milk during production and handling mainly by nonsterile equipment; and that, when this source is eliminated, typical vigorous *S. lactis* cells may be absent from the milk with consequent abnormal souring.

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